Appendix 5

Standard Operating Procedures and Scientific Literature Review for Population Growth Suppression Methods

*A **note to readers**: This appendix contains standard operating procedures (SOP), including implementation and monitoring requirements, associated with the various population growth suppression methods contemplated in the Proposed Action, including the use of fertility control vaccines and intrauterine devices (IUD). Following these SOPs, the appendix includes a detailed scientific literature review of the effects of the fertility control vaccines, sex ratio adjustment, and IUDs included as part of the Proposed Action.*

SOPs for Population Growth Suppression Methods

SOPs common to all population growth suppression methods
Identification
Animals intended for treatment must be clearly, individually identifiable to allow for positive identification during subsequent management activities. For captured animals, marking for identification may be accomplished by marking each individual with a freeze mark on the hip and/ or neck and a microchip in the nuchal ligament. In some cases, identification may be accomplished based by cataloguing markings that make animals uniquely identifiable. Such animals may be photographed using a telephoto lens and high-quality digital camera as a record of treated individuals.

Safety
Safety for both humans and animals is the primary consideration in all elements of population growth suppression use. Administration of any vaccine or IUD must follow all safety guidance and label guidelines on applicable EPA labeling.

Monitoring and Tracking of Treatments
1. Estimation of population size and growth rates (in most cases, using aerial surveys) should be conducted periodically after treatments.
2. Population growth rates of some herds selected for intensive monitoring may be estimated every year post-treatment using aerial surveys. If, during routine HMA field monitoring (on-the-ground), data describing adult to foal ratios can be collected, these data should also be shared with HQ-261.
3. Field applicators should record all pertinent data relating to identification of treated animals (including photographs if animals are not freeze-marked) and date of treatment, lot number(s) of the vaccine, quantity of vaccine issued, the quantity used, the date of vaccination, disposition of any unused vaccine, the date disposed, the number of treated mares by HMA, field office, and State along with the microchip numbers and freeze-mark(s) applied by HMA and date. A summary narrative and data sheets will be forwarded to HQ-261 annually (Reno, Nevada). A copy of the form and data sheets and any photos taken should be maintained at the field office.

4. HQ-261 will maintain records sent from field offices, on the quantity of PZP issued, the quantity used, disposition of any unused PZP, the number of treated mares by HMA, field office, and State along with the freeze-mark(s) applied by HMA and date.

SOPs common to all fertility control vaccine types

Injection Site
For hand-injection, delivery of the vaccine should be by intramuscular injection, while the animal is standing still, into the left or right side, above the imaginary line that connects the point of the hip (hook bone) and the point of the buttocks (pin bone): this is the hip / upper gluteal area. For dart-based injection, delivery of the vaccine should be by intramuscular injection, while the animal is standing still, into the left or right thigh areas (lower gluteal / biceps femoralis).

SOPs for one-year liquid PZP vaccine (ZonaStat-H)

ZonaStat-H vaccine (Science and Conservation Center, Billings, MT) would be administered through hand-injection or darting by trained BLM personnel or collaborating partners only. At present, the only PZP vaccine for dart-based delivery in BLM-managed wild horses or burros is ZonaStat-H. For any darting operation, the designated personnel must have successfully completed a nationally recognized wildlife darting course and who have documented and successful experience darting wildlife under field conditions. Until the day of its use, ZonaStat-H must be kept frozen.

Animals that have never been treated with a PZP vaccine would receive 0.5 cc of PZP vaccine emulsified with 0.5 cc of Freund's Modified Adjuvant (FMA). Animals identified for re-treatment receive 0.5 cc of the PZP vaccine emulsified with 0.5 cc of Freund's Incomplete Adjuvant (FIA).
Hand-injection of liquid PZP vaccine would be by intramuscular injection into the gluteal muscles while the animal is restrained in a working chute. The vaccine would be injected into the left hind quarters of the animal, above the imaginary line that connects the point of the hip (hook bone) and the point of the buttocks (pin bone).

For Hand-injection, delivery of the vaccine would be by intramuscular injection into the left or right buttocks and thigh muscles (gluteals, biceps femoris) while the animal is standing still.

Application of ZonaStat-H via Darting
Only designated darters would prepare the emulsion. Vaccine-adjuvant emulsion would be loaded into darts at the darting site and delivered by means of a projector gun.

No attempt to dart should be taken when other persons are within a 100-m radius of the target animal. The Dan Inject gun should not be used at ranges in excess of 30 m while the Pneu-Dart gun should not be used over 50 m.

No attempts would be taken in high wind (greater than 15 mph) or when the animal is standing at an angle where the dart could miss the target area and hit the flank or rib cage. The ideal is when the dart would strike the skin of the animal at a 90° angle.

If a loaded dart is not used within two hours of the time of loading, the contents would be transferred to a new dart before attempting another animal. If the dart is not used before the end of the day, it would be stored under refrigeration and the contents transferred to another dart the next day, for a maximum of one transfer (discard contents if not used on the second day). Refrigerated darts would not be used in the field.

A darting team should include two people. The second person is responsible for locating fired darts. The second person should also be responsible for identifying the animal and keeping onlookers at a safe distance.

To the extent possible, all darting would be carried out in a discrete manner. However, if darting is to be done within view of non-participants or members of the public, an explanation of the nature of the project would be carried out either immediately before or after the darting.

Attempts will be made to recover all darts. To the extent possible, all darts which are discharged and drop from the target animal at the darting site would be recovered before another darting occurs. In exceptional situations, the site of a lost dart may be noted and marked, and recovery efforts made at a later time. All discharged darts would be examined after recovery in order to determine if the charge fired and the plunger fully expelled the vaccine. Personnel conducting darting operations should be equipped with a two-way radio or cell phone to provide a communications link with a project veterinarian for advice and/or assistance. In the event of a veterinary emergency, darting personnel would immediately contact the project veterinarian, providing all available information concerning the nature and location of the incident.

In the event that a dart strikes a bone or imbeds in soft tissue and does not dislodge, the darter would follow the affected animal until the dart falls out or the animal can no longer be found. The darter would be responsible for daily observation of the animal until the situation is resolved.

SOPs for application of PZP-22 pelleted vaccine
PZP-22 pelleted vaccine treatment would be administered only by trained BLM personnel or designated partners. A treatment of PZP-22 is comprised of two separate injections: (1) a liquid dose of PZP vaccine (equivalent to one dose of ZonaStat-H) is administered using an 18-gauge needle primarily by hand injection; and (2) the pellets are preloaded into a 14-gauge needle. For animals constrained in a working chute, these are delivered using a modified syringe and jabstick to inject the pellets into the gluteal muscles of the animals being returned to the range. The pellets are intended to release PZP over time. Until the day of its use, the liquid portion of PZP-22 must be kept frozen.

At this time, delivery of PZP-22 treatment would only be by intramuscular injection into the gluteal muscles while the animal is restrained in a working chute. The primer would consist of 0.5 cc of liquid PZP emulsified with 0.5 cc of adjuvant. Animals that have never been treated with a PZP vaccine would receive 0.5 cc of PZP vaccine emulsified with 0.5 cc of Freund's Modified Adjuvant (FMA). Animals identified for re-treatment receive 0.5 cc of the PZP vaccine emulsified with 0.5 cc of Freund's Incomplete Adjuvant (FIA). The syringe with PZP vaccine pellets would be loaded into the jabstick for the second injection. With each injection, the liquid

or pellets would be injected into the left hind quarters of the animal, above the imaginary line that connects the point of the hip (hook bone) and the point of the buttocks (pin bone). In the future, the PZP-22 treatment may be administered remotely using an approved long range darting protocol and delivery system if and when BLM has determined that the technology has been proven safe and effective for use.

SOPs for GonaCon-Equine vaccine
GonaCon-Equine vaccine (USDA Pocatello Storage Depot, Pocatello, ID; Spay First!, Inc., Oklahoma City, OK) is distributed as preloaded doses (2 mL) in labeled syringes. Upon receipt, the vaccine should be kept refrigerated (4° C) until use. Do not freeze GonaCon-Equine. The vaccine has a 6-month shelf-life from the time of production and the expiration date will be noted on each syringe that is provided. For initial and booster treatments, mares would ideally receive 2.0 ml of GonaCon-Equine.

Administering GonaCon Vaccine by Hand-Injection.
Experience has demonstrated that only 1.8 ml of vaccine can typically be loaded into 2 cc darts, and this dose has proven successful. The calculations below reflect a 1.8 ml dose. For hand-injection, delivery of the vaccine should be by intramuscular injection, while the animal is standing still, into the left or right side, above the imaginary line that connects the point of the hip (hook bone) and the point of the buttocks (pin bone): this is the hip / upper gluteal area. A booster vaccine may be administered after the first injection to improve efficacy of the product over subsequent years.

Application of GonaCon-Equine via Darting
General practice guidelines for darting operations, as noted above for dart-delivery of ZonaStat-H, should be followed for dart-delivery of GonaCon-Equine.

Wearing latex gloves, the applicator numbers darts and loads the numbered darts with vaccine by attaching a loading needle (7.62 cm; provided by dart manufacturer) to the syringe containing vaccine and placing the needle into the cannula of the dart to the fullest depth possible. Slowly depress the syringe plunger and begin filling the dart. Periodically, tap the dart on a hard surface to dislodge air bubbles trapped within the vaccine. Due to the viscous nature of the fluid, air entrapment typically results in a maximum of approximately 1.8 ml of vaccine being loaded in the dart. The dart is filled to maximum once a small amount of the vaccine can be seen at the tri-ports.

Important! Do not load and refrigerate darts the night before application. When exposed to moisture and condensation, the edges of gel barbs soften, begin to dissolve, and will not hold the dart in the muscle tissue long enough for full injection of the vaccine. The dart needs to remain in the muscle tissue for a minimum of 1 minute to achieve dependable full injection. Sharp gel barbs are critical.

Darts should be weighed to the nearest hundredth gram by electronic scale when empty, when loaded with vaccine, and after discharge, to ensure that 90% (1.62 ml) of the vaccine has been injected. GonaCon-Equine weighs 0.95 grams/mL, so animals should receive 1.54 grams of vaccine to be considered treated. Animals receiving <50% should be darted with another full

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dose; those receiving >50% but <90% should receive a half dose (1 ml). All darts should be weighed to verify a combination of ≥1.62 ml has been administered. Therefore, every effort should be made to recover darts after they have fallen from animals.

Although infrequent, dart injections can result in partial injections of the vaccine, and shots are missed. As a precaution, it is recommended that extra doses of the vaccine be ordered to accommodate failed delivery (which may be as high as ~15 %). To determine the amount of vaccine delivered, the dart must be weighed before loading, and before and after delivery in the field. The scale should be sensitive to 0.01 grams or less, and accurate to 0.05 g or less.

For best results, darts with a gel barb should be used. (i.e., 2 cc Pneu-Dart brand darts configured with Slow-inject technology, 3.81 cm long 14 ga.tri-port needles, and gel collars positioned 1.27 cm ahead of the ferrule). One can expect updates in optimal dart configuration, pending results of research and field applications.

Darts (configured specifically as described above) can be loaded in the field and stored in a cooler prior to application. Darts loaded, but not used can be maintained in dry conditions at about 4° C and used the next day, but do not store in any refrigerator or container likely to cause condensation, which can compromise the gel barbs.

SOPs for Insertion of Y-shaped Silicone IUD for Feral Horses
Background
Mares must be open. A veterinarian must determine pregnancy status via palpation or ultrasound. Ultrasound should be used as necessary to confirm open status of mares down to at least 14 days for those that have recently been with stallions. For mares segregated from stallions, this determination may be made at an earlier time when mares are identified as candidates for treatment, or immediately prior to IUD insertion. Pregnant mares should not receive an IUD.

Preparation
IUDs must be clean and sterile. Sterilize IUDs with a low-temperature sterilization system, such as Sterrad. The Introducer is two PVC pipes. The exterior pipe is a 29" length of ½" diameter pipe, sanded smooth at one end, then heat-treated to smooth its curvature further (Fig. 1). The IUD will be placed into this smoothed end of the exterior pipe. The interior pipe is a 29 ½" long, ¼" riser tube (of the kind used to connect water lines to sinks), with one end slightly flared out to fit more snugly inside the exterior pipe (Fig. 1), and a plastic stopper attached to the other end (Fig. 2).

Figure 1. Interior and exterior pipes (unassembled), showing the ends that go into the mare

Figure 2. Interior pipe shown within exterior pipe. After the introducer is 4" beyond the os, the stopper is pushed forward (outside the mare), causing the IUD to be pushed out from the exterior pipe.

Introducers should be sterilized in Benz-all cold steriliant, or similar. Do not use iodine-based sterilant solution. A suitable container for sterilant can be a large diameter (i.e., 2") PVC pipe with one end sealed and one end removable.

Prepare the IUD: Lubricate with sterile veterinary lube, and insert into the introducer. The central stem of the IUD goes in first (Fig. 3).

Figure 3. Insert the stem end of the IUD into the exterior pipe.

Fold the two 'legs' of the IUD, and push the IUD further into the introducer, until just the bulbous ends are showing (Fig. 4).
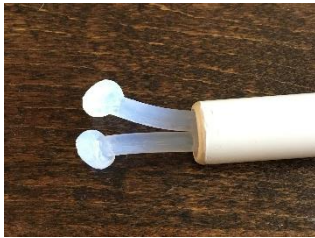

Figure 4. Insert the IUD until just the tips of the 'legs' are showing.

Restraint and Medication

The mare should be restrained in a padded squeeze chute to provide access to the rear end of the animal, but with a solid lower back door, or thick wood panel, for veterinarian safety. Only a veterinarian shall oversee this procedure and insert IUDs. Some practitioners may choose to provide sedation. If so, when the mare's head starts to droop, it may be advisable to tie the tail up to prevent risk of the animal sitting down on the veterinarian's arm (i.e., double half hitch, then tie tail to the bar above the animal).

Some practitioners may choose to provide a dose of long-acting progesterone to aid in IUD retention. Example dosage: 5mL of BioRelease LA Progesterone 300 mg/mL (BET labs, Lexington KY), *or* long-acting Altrenogest). No other intrauterine treatments of any kind should be administered at the time of IUD insertion.

Insertion Procedure
1. Prep clean the perineal area.
2. Lubricate the veterinarian's sleeved arm and the Introducer+IUD.
3. Carry the introducer (IUD-end-first) into the vagina.

4. Dilate the cervix and gently move the tip of the introducer past the cervix.
5. Advance the end of the 1/2" PVC pipe about 4 inches past the internal os of the cervix.
6. Hold the exterior pipe in place, but push the stopper of the interior pipe forward, causing the IUD to be pushed out of the exterior pipe, into the uterus.
7. Placing a finger into the cervical lumen just as the introducer tube is removed from the external os allows the veterinarian to know that the IUD is left in the uterus, and not dragged back into or past the cervix.
8. Remove the introducer from the animal, untie the tail.

Mares that have received an IUD should be observed closely for signs of discharge or discomfort for 24 hours following insertion after which they may be released back to the range.

Label for Y-Shaped Silicone IUD for Feral Horses

Y-Shaped Silicone IUD for Feral Horses

The *Y-Shaped Silicone IUD for Feral Horses* is an intrauterine device (IUD) comprised solely of medical-grade, inert, silicone that is suitable for use in female feral horses (free-roaming or "wild" *Equus caballus*). Intended users include government agencies with feral horses in their management purview, Native American tribes that have management authority over feral horses, and authorized feral horse care or rescue sanctuaries that manage feral horses in a free ranging environment.

The *Y-Shaped Silicone IUD for Feral Horses* can mitigate or reduce feral horse population growth rates because these IUDs can provide potentially reversible fertility control for female feral horses. This IUD prevents pregnancy by its physical presence in the mare's uterus as long as the IUD stays in place. In trials, approximately 75% of mares living and breeding with fertile stallions retained the *Y-Shaped Silicone IUD for Feral Horses* over two breeding seasons. None of the mares that kept their IUDs became pregnant during an experimental trial. After IUD removal, the majority of mares returned to fertility.

Directions for Use:

The *Y-Shaped Silicone IUD for Feral Horses* is to be placed in the uterus of feral horse mares by a veterinarian. The *Y-Shaped Silicone IUD for Feral Horses* is intended for use in feral mares that are at least approximately 1 year old, where age is determined based on available evidence, such as tooth eruption pattern.

IUDs must be sterilized before use. The IUD is inserted into the uterus using a thin, tubular applicator, similar to a shielded culture tube commonly used in equine reproductive veterinary medicine, in a manner similar to methods used for uterine culture of domestic mares. Feral mares with IUDs should be individually marked and identified (i.e., with an RFID microchip, or via visible freeze-brand on the hip or neck).

Caution:

These IUDs are only to be used in mares that are confirmed to be not pregnant. Checking pregnancy status can be accomplished by methods such as a transrectal palpation and/or ultrasound performed by a veterinarian. If a *Y-Shaped Silicone IUD for Feral Horses* is inserted in the uterus of a pregnant mare, it may cause the pregnancy to terminate, and the IUD to be expelled.

Manufactured for:

U.S. Bureau of Land Management (97949)
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EPA Est.: 97628-MI-1

Scientific Literature Review of Effects of Fertility Control Vaccines and Sex Ratio Adjustments

Various forms of fertility control can be used in wild horses and wild burros, with the goals of maintaining herds at or near AML, reducing fertility rates, and reducing the frequency of gathers and removals. The Wild Free-Roaming Horses and Burros Act of 1971 (WFRHBA) specifically provides for contraception and sterilization (16 U.S.C. 1333 section 3(b)(1)). Fertility control measures have been shown to be a cost-effective and humane treatment to slow increases in wild horse populations or, when used in combination with gathers, to reduce horse population size (Bartholow, 2004; de Seve and Boyles-Griffin, 2013; Fonner and Bohara, 2017). Although fertility control treatments may be associated with a number of potential physiological, behavioral, demographic, and genetic effects, those impacts are generally minor and transient, do not prevent overall maintenance of a self-sustaining population, and do not generally outweigh the potential benefits of using contraceptive treatments in situations where it is a management goal to reduce population growth rates (Garrott and Oli, 2013).

An extensive body of peer-reviewed scientific literature details the impacts of fertility control methods on wild horses and burros. No finding of excess animals is required for BLM to pursue contraception in wild horses or wild burros, but National Environmental Policy Act (NEPA) analysis has been required. This review focuses on peer-reviewed scientific literature. The summary that follows first examines effects of fertility control vaccine use in mares then of sex ratio adjustment. This review does not examine effects of spaying and neutering. Cited studies are generally limited to those involving horses and burros, except where including studies on other species helps in making inferences about physiological or behavioral questions not yet addressed in horses or burros specifically. While most studies reviewed here refer to horses, burros are extremely similar in terms of physiology, such that expected effects are comparable, except where differences between the species are noted.

On the whole, the identified impacts are generally transient and affect primarily the individuals treated. Fertility control that affects individual horses and burros does not prevent BLM from ensuring that there will be self-sustaining populations of wild horses and burros in single herd management areas (HMAs), in complexes of HMAs, and at regional scales of multiple HMAs and complexes. Under the WFRHBA, BLM is charged with maintaining self-reproducing populations of wild horses and burros. The National Research Council of the National Academy of Sciences (NAS) report (2013) encouraged BLM to manage wild horses and burros at the spatial scale of "metapopulations" – that is, across multiple HMAs and complexes in a region. In fact, many HMAs have historical and ongoing genetic and demographic connections with other HMAs, and BLM routinely moves animals from one to another to improve local herd traits and maintain high genetic diversity. The NAS report (2013) includes information (pairwise genetic 'fixation index' values for sampled WH&B herds) confirming that wild horses and burros in the vast majority of HMAs are genetically similar to animals in multiple other HMAs.

All fertility control methods affect the behavior and physiology of treated animals (NAS, 2013), and are associated with potential risks and benefits, including effects of handling, frequency of handling, physiological effects, behavioral effects, and reduced population growth rates (Hampton et al., 2015). Contraception alone does not remove excess horses from an HMA's

population, so one or more gathers are usually needed in order to bring the herd down to a level close to AML. Horses are long-lived, potentially reaching 20 years of age or more in the wild. Except in cases where extremely high fractions of mares are rendered infertile over long time periods of (i.e., 10 or more years), fertility control methods such as immunocontraceptive vaccines and sex ratio adjustment are not very effective at reducing population growth rates to the point where births equal deaths in a herd. However, even more modest fertility control activities can reduce the frequency of horse gather activities, and costs to taxpayers. Bartholow (2007) concluded that the application of 2-year or 3-year contraceptives to wild mares could reduce operational costs in a project area by 12-20%, or up to 30% in carefully planned population management programs. Because applying contraception to horses requires capturing and handling, the risks and costs associated with capture and handling of horses may be comparable to those of gathering for removal, but with expectedly lower adoption and long-term holding costs. Population growth suppression becomes less expensive if fertility control is long-lasting (Hobbs et al., 2000).

In the context of BLM wild horse and burro management, fertility control vaccines and sex ratio adjustment rely on reducing the number of reproducing females. Taking into consideration available literature on the subject, the NAS concluded in their 2013 report that forms of fertility control vaccines were two of the three "most promising" available methods for contraception in wild horses and burros (NAS, 2013). That report also noted that sex ratio adjustments where herds have approximately 60% males and 40% females can expect lower annual growth rates, simply as a result of having a lower number of reproducing females.

Fertility Control Vaccines
Fertility control vaccines (also known as (immunocontraceptives) meet BLM requirements for safety to mares and the environment (EPA, 2009a, 2012). Because they work by causing an immune response in treated animals, there is no risk of hormones or toxins being taken into the food chain when a treated mare dies. The BLM and other land managers have mainly used three fertility control vaccine formulations for fertility control of wild horse mares on the range: ZonaStat-H, PZP-22, and GonaCon-Equine. As other formulations become available, they may be applied in the future.

In any vaccine, the antigen is the stimulant to which the body responds by making antigen-specific antibodies. Those antibodies then signal to the body that a foreign molecule is present, initiating an immune response that removes the molecule or cell. Adjuvants are additional substances that are included in vaccines to elevate the level of immune response. Adjuvants help to incite recruitment of lymphocytes and other immune cells which foster a long-lasting immune response that is specific to the antigen.

Liquid emulsion vaccines can be injected by hand or remotely administered in the field using a pneumatic dart (Roelle and Ransom, 2009; Rutberg et al., 2017; McCann et al., 2017) in cases where mares are relatively approachable. Use of remotely delivered (dart-delivered) vaccine is generally limited to populations where individual animals can be accurately identified and repeatedly approached within 50 m (BLM, 2010). Booster doses can be safely administered by hand or by dart. Even with repeated booster treatments of the vaccines, it is expected that most mares would eventually return to fertility, though some individual mares treated repeatedly may

remain infertile. Once the herd size in a project area is at AML and population growth seems to be stabilized, BLM can make adaptive determinations as to the required frequency of new and booster treatments.

BLM has followed SOPs for fertility control vaccine application (BLM IM 2009-090). Herds selected for fertility control vaccine use should have annual growth rates over 5%, have a herd size over 50 animals, and have a target rate of treatment of between 50% and 90% of female wild horses or burros. The IM requires that treated mares be identifiable via a visible freeze brand or individual color markings, so that their vaccination history can be known. The IM calls for follow-up population surveys to determine the realized annual growth rate in herds treated with fertility control vaccines.

Vaccine Formulations: Porcine Zona Pellucida (PZP)
PZP vaccines have been used on dozens of horse herds by the National Park Service, U.S. Forest Service, BLM, and Native American Tribes and PZP vaccine use is approved for free-ranging wild and feral horse herds in the United States (EPA, 2012). PZP use can reduce or eliminate the need for gathers and removals, if very high fractions of mares are treated over a very long time period (Turner et al., 1997). PZP vaccines have been used extensively in wild horses (NAS, 2013), and in feral burros on Caribbean islands (Turner et al., 1996; French et al., 2017). PZP vaccine formulations are produced as ZonaStat-H, an EPA-registered commercial product (EPA, 2012; SCC, 2015), as PZP-22, which is a formulation of PZP in polymer pellets that can lead to a longer immune response (Turner et al., 2002; Rutberg et al., 2017), and as Spayvac, where the PZP protein is enveloped in liposomes (Killian et al., 2008; Roelle et al., 2017; Bechert and Fraker, 2018). 'Native' PZP proteins can be purified from pig ovaries (Liu et al., 1989). Recombinant ZP proteins may be produced with molecular techniques (Gupta and Minhas, 2017; Joonè et al., 2017a; Nolan et al., 2018a).

When advisories on the product label (EPA, 2015) are followed, the product is safe for users and the environment (EPA, 2012). In keeping with the EPA registration for ZonaStat-H (EPA. 2012; reg. no. 86833-1), certification through the Science and Conservation Center in Billings Montana is required to apply that vaccine to equids.

For maximum effectiveness, PZP is administered within the December to February timeframe. When applying ZonaStat-H, first the primer with modified Freund's Complete adjuvant is given and then the booster with Freund's Incomplete adjuvant is given 2-6 weeks later. Preferably, the timing of the booster dose is at least 1-2 weeks prior to the onset of breeding activity. Following the initial 2 inoculations, only annual boosters are required. For the PZP-22 formulation, each released mare would receive a single dose of the two-year PZP contraceptive vaccine at the same time as a dose of the liquid PZP vaccine with modified Freund's Complete adjuvant. The pellets are applied to the mare with a large gauge needle and jab-stick into the hip. Although PZP-22 pellets have been delivered via darting in trial studies (Rutberg et al., 2017; Carey et al., 2019), BLM does not plan to use darting for PZP-22 delivery until there is more demonstration that PZP-22 can be reliably delivered via dart.

Vaccine Formulations: Gonadotropin Releasing Hormone (GnRH)
GonaCon (which is produced under the trade name GonaCon-Equine for use in feral horses and

burros) is approved for use by authorized Federal, State, Tribal, public, and private personnel for application to free-ranging wild horse and burro herds in the United States (EPA, 2013, 2015). GonaCon has been used on feral horses in Theodore Roosevelt National Park and on wild horses administered by BLM (BLM, 2015). GonaCon has been produced by USDA-APHIS (Fort Collins, Colorado) in several different formulations, the history of which is reviewed by Miller et al. (2013). GonaCon vaccines present the recipient with hundreds of copies of GnRH as peptides on the surface of a linked protein that is naturally antigenic because it comes from invertebrate hemocyanin (Miller et al., 2013). Early GonaCon formulations linked many copies of GnRH to a protein from the keyhole limpet (GonaCon-KHL), but more recently produced formulations where the GnRH antigen is linked to a protein from the blue mussel (GonaCon-B) proved less expensive and more effective (Miller et al., 2008). GonaCon-Equine is in the category of GonaCon-B vaccines.

As with other contraceptives applied to wild horses, the long-term goal of GonaCon-Equine use is to reduce or eliminate the need for gathers and removals (NAS, 2013). GonaCon-Equine contraceptive vaccine is an EPA-approved pesticide (EPA, 2009a) that is relatively inexpensive, meets BLM requirements for safety to mares and the environment, and is produced in a USDA-APHIS laboratory. GonaCon is a pharmaceutical-grade vaccine, including aseptic manufacturing technique to deliver a sterile vaccine product (Miller et al., 2013). If stored at 4° C, the shelf life is 6 months (Miller et al., 2013).

Miller et al. (2013) reviewed the vaccine environmental safety and toxicity. When advisories on the product label (EPA, 2015) are followed, the product is safe for users and the environment (EPA, 2009b). EPA waived a number of tests prior to registering the vaccine, because GonaCon was deemed to pose low risks to the environment, so long as the product label is followed (Wang-Cahill et al., *in press*).

GonaCon-Equine can safely be reapplied as necessary to control the population growth rate; booster dose effects may lead to increased effectiveness of contraception, which is generally the intent. Even after booster treatment of GonaCon-Equine, it is expected that most, if not all, mares would return to fertility at some point. Although the exact timing for the return to fertility in mares boosted more than once with GonaCon-Equine has not been quantified, a prolonged return to fertility would be consistent with the desired effect of using GonaCon (e.g., effective contraception).

The adjuvant used in GonaCon, Adjuvac, generally leads to a milder reaction than Freund's Complete Adjuvant (Powers et al., 2011). Adjuvac contains a small number of killed Mycobacterium avium cells (Miller et al., 2008; Miller et al., 2013). The antigen and adjuvant are emulsified in mineral oil, such that they are not all presented to the immune system right after injection. It is thought that the mineral oil emulsion leads to a 'depot effect' that is associated with slow or sustained release of the antigen, and a resulting longer-lasting immune response (Miller et al., 2013). Miller et al. (2008, 2013) have speculated that, in cases where memory-B leukocytes are protected in immune complexes in the lymphatic system, it can lead to years of immune response. Increased doses of vaccine may lead to stronger immune reactions, but only to a certain point; when Yoder and Miller (2010) tested varying doses of GonaCon in prairie dogs, antibody responses to the 200μg and 400μg doses were equal to each other but were both higher

than in response to a 100μg dose.

GonaCon-Equine is one of several vaccines that have been engineered to create an immune response to the gonadotropin releasing hormone peptide (GnRH). GnRH is a small peptide that plays an important role in signaling the production of other hormones involved in reproduction in both sexes. When combined with an adjuvant, a GnRH vaccine stimulates a persistent immune response resulting in prolonged antibody production against GnRH, the carrier protein, and the adjuvant (Miller et al., 2008). The most direct result of successful GnRH vaccination is that it has the effect of decreasing the level of GnRH signaling in the body, as evidenced by a drop in luteinizing hormone levels, and a cessation of ovulation.

GnRH is highly conserved across mammalian taxa, so some inferences about the mechanism and effects of GonaCon-Equine in horses can be made from studies that used different anti-GnRH vaccines, in horses and other taxa. Other commercially available anti-GnRH vaccines include: Improvac (Imboden et al., 2006; Botha et al., 2008; Janett et al., 2009a; Janett et al., 2009b; Schulman et al., 2013; Dalmau et al., 2015; Nolan et al., 2018c), made in South Africa; Equity (Elhay et al. 2007), made in Australia; Improvest, for use in swine (Bohrer et al., 2014); Repro-BLOC (Boedeker et al., 2011); and Bopriva, for use in cows (Balet et al., 2014). Of these, GonaCon-Equine, Improvac, and Equity are specifically intended for horses. Other anti-GnRH vaccine formulations have also been tested, but did not become trademarked products (e.g., Goodloe, 1991; Dalin et al., 2002; Stout et al., 2003; Donovan et al., 2013; Schaut et al., 2018; Yao et al., 2018). The effectiveness and side-effects of these various anti-GnRH vaccines may not be the same as would be expected from GonaCon-Equine use in horses. Results could differ as a result of differences in the preparation of the GnRH antigen, and the choice of adjuvant used to stimulate the immune response. For some formulations of anti-GnRH vaccines, a booster dose is required to elicit a contraceptive response, though GonaCon can cause short-term contraception in a fraction of treated animals from one dose (Powers et al., 2011; Gionfriddo et al., 2011a; Baker et al., 2013; Miller et al., 2013).

GonaCon can provide multiple years of infertility in several wild ungulate species, including horses (Killian et al., 2008; Gray et al., 2010). The lack of estrus cycling that results from successful GonaCon vaccination has been compared to typical winter period of anoestrus in open mares. As anti-GnRH antibodies decline over time, concentrations of available endogenous GnRH increase and treated animals usually regain fertility (Power et al., 2011).

Females that are successfully contracepted by GnRH vaccination enter a state similar to anestrus, have a lack of or incomplete follicle maturation, and no ovarian cycling (Botha et al., 2008; Nolan et al., 2018c). A leading hypothesis is that anti-GnRH antibodies bind GnRH in the hypothalamus – pituitary 'portal vessels,' preventing GnRH from binding to GnRH-specific binding sites on gonadotroph cells in the pituitary, thereby limiting the production of gonadotropin hormones, particularly luteinizing hormone (LH) and, to a lesser degree, follicle-stimulating hormone (FSH) (Powers et al., 2011; NAS, 2013). This reduction in LH (and FSH), and a corresponding lack of ovulation, has been measured in response to treatment with anti-GnRH vaccines (Boedeker et al., 2011; Garza et al., 1986).

Females successfully treated with anti-GnRH vaccines have reduced progesterone levels (Garza et al. 1986; Stout et al., 2003; Imboden et al., 2006; Elhay, 2007; Botha et al., 2008; Killian et al., 2008; Miller et al., 2008; Janett et al., 2009; Schulman et al., 2013; Balet et al. 2014; Dalmau et al., 2015) and β-17 estradiol levels (Elhay et al., 2007), but no great decrease in estrogen levels (Balet et al., 2014). Reductions in progesterone do not occur immediately after the primer dose but can take several weeks or months to develop (Elhay et al., 2007; Botha et al., 2008; Schulman et al., 2013; Dalmau et al., 2015). This indicates that ovulation is not occurring and corpora lutea, formed from post-ovulation follicular tissue, are not being established.

Antibody titer measurements are proximate measures of the antibody concentration in the blood specific to a given antigen. Anti-GnRH titers generally correlate with a suppressed reproduction system (Gionfriddo et al., 2011a; Powers et al., 2011). Various studies have attempted to identify a relationship between anti-GnRH titer levels and infertility, but that relationship has not been universally predictable or consistent. The time length that titer levels stay high appears to correlate with the length of suppressed reproduction (Dalin et al., 2002; Levy et al., 2011; Donovan et al., 2013; Powers et al., 2011). For example, Goodloe (1991) noted that mares did produce elevated titers and had suppressed follicular development for 11-13 weeks after treatment, but that all treated mares ovulated after the titer levels declined. Similarly, Elhay (2007) found that high initial titers correlated with longer-lasting ovarian and behavioral anoestrus. However, Powers et al. (2011) did not identify a threshold level of titer that was consistently indicative of suppressed reproduction despite seeing a strong correlation between antibody concentration and infertility, nor did Schulman et al. (2013) find a clear relationship between titer levels and mare acyclicity.

In many cases, young animals appear to have higher immune responses, and stronger contraceptive effects of anti-GnRH vaccines than older animals (Brown et al., 1994; Curtis et al., 2001; Stout et al., 2003; Schulman et al., 2013). Vaccinating with GonaCon at too young an age, though, may prevent effectiveness; Gionfriddo et al. (2011a) observed weak effects in 3- to 4-month-old fawns. It has not been possible to predict which individuals of a given age class will have long-lasting immune responses to the GonaCon vaccine. Gray (2010) noted that mares in poor body condition tended to have lower contraceptive efficacy in response to GonaCon-B. Miller et al. (2013) suggested that higher parasite loads might have explained a lower immune response in free-roaming horses than had been observed in a captive trial. At this time, it is unclear what the most important factors affecting efficacy are.

Several studies have monitored animal health after immunization against GnRH. GonaCon treated mares did not have any measurable difference in uterine edema (Killian, 2006, 2008). Powers et al. (2011, 2013) noted no differences in blood chemistry except a mildly elevated fibrinogen level in some GonaCon treated elk. In that study, one sham-treated elk and one GonaCon treated elk each developed leukocytosis, suggesting that there may have been a causal link between the adjuvant and the effect. Curtis et al. (2008) found persistent granulomas at GonaCon-KHL injection sites three years after injection, and reduced ovary weights in treated females. Yoder and Miller (2010) found no difference in blood chemistry between GonaCon treated and control prairie dogs. One of 15 GonaCon treated cats died without explanation, and with no determination about cause of death possible based on necropsy or histology (Levy et al., 2011). Other anti-GnRH vaccine formulations have led to no detectable adverse effects (in

elephants; Boedeker et al., 2011), though Imboden et al. (2006) speculated that young, treated animals might conceivably have impaired hypothalamic or pituitary function.

Kirkpatrick et al. (2011) raised concerns that anti-GnRH vaccines could lead to adverse effects in other organ systems outside the reproductive system. GnRH receptors have been identified in tissues outside of the pituitary system, including in the testes and placenta (Khodr and Siler-Khodr, 1980), ovary (Hsueh and Erickson, 1979), bladder (Coit et al., 2009), heart (Dong et al., 2011), and central nervous system, so it is plausible that reductions in circulating GnRH levels could inhibit physiological processes in those organ systems. Kirkpatrick et al. (2011) noted elevated cardiological risks to human patients taking GnRH agonists (such as leuprolide), but the NAS (2013) concluded that the mechanism and results of GnRH agonists would be expected to be different from that of anti-GnRH antibodies; the former flood GnRH receptors, while the latter deprive receptors of GnRH.

Reversibility and Effects on Ovaries: PZP Vaccines
In most cases, PZP contraception appears to be temporary and reversible, with most treated mares returning to fertility over time (Kirkpatrick and Turner, 2002). The ZonaStat-H formulation of the vaccine tends to confer only one year of efficacy per dose. Some studies have found that a PZP vaccine in long-lasting pellets (PZP-22) can confer multiple years of contraception (Turner et al., 2007), particularly when boostered with subsequent PZP vaccination (Rutberg et al., 2017). Other trial data, though, indicate that the pelleted vaccine may only be effective for one year (J. Turner, University of Toledo, Personal Communication to BLM).

The purpose of applying PZP vaccine treatment is to prevent mares from conceiving foals, but BLM acknowledges that long-term infertility, or permanent sterility, could be a result for some number of individual wild horses receiving PZP vaccinations. The rate of long-term or permanent sterility following vaccinations with PZP is hard to predict for individual horses, but that outcome appears to increase in likelihood as the number of doses increases (Kirkpatrick and Turner, 2002). Permanent sterility for mares treated consecutively in each of 5-7 years was observed by Nuñez et al. (2010, 2017). In a graduate thesis, Knight (2014) suggested that repeated treatment with as few as three to four years of PZP treatment may lead to longer-term sterility, and that sterility may result from PZP treatment before puberty. Repeated treatment with PZP led long-term infertility in Przewalski's horses receiving as few as one PZP booster dose (Feh, 2012). However, even if some number of mares become sterile as a result of PZP treatment, that potential result would be consistent with the contraceptive purpose that motivates BLM's potential use of the vaccine.

In some number of individual mares, PZP vaccination may cause direct effects on ovaries (Gray and Cameron, 2010; Joonè et al., 2017b; Joonè et al., 2017c; Joonè et al., 2017d; Nolan et al., 2018b). Joonè et al. (2017a) noted reversible effects on ovaries in mares treated with one primer dose and booster dose. Joonè et al. (2017c) and Nolan et al. (2018b) documented decreased anti-Mullerian hormone (AMH) levels in mares treated with native or recombinant PZP vaccines; AMH levels are thought to be an indicator of ovarian function. Bechert et al. (2013) found that ovarian function was affected by the SpayVac PZP vaccination, but that there were no effects on other organ systems. Mask et al. (2015) demonstrated that equine antibodies that resulted from SpayVac immunization could bind to oocytes, ZP proteins, follicular tissues, and ovarian tissues.

It is possible that result is specific to the immune response to SpayVac, which may have lower PZP purity than ZonaStat or PZP-22 (Hall et al. 2016).

However, in studies with native ZP proteins and recombinant ZP proteins, Joonè et al. (2017a) found transient effects on ovaries after PZP vaccination in some treated mares; normal estrus cycling had resumed 10 months after the last treatment. SpayVac is a patented formulation of PZP in liposomes that led to multiple years of infertility in some breeding trials (Killian et al., 2008; Roelle et al., 2017; Bechert and Fraker, 2018), but unacceptably poor efficacy in a subsequent trial (Kane, 2018). Kirkpatrick et al. (1992) noted effects on horse ovaries after three years of treatment with PZP. Observations at Assateague Island National Seashore indicated that the more times a mare is consecutively treated, the longer the time lag before fertility returns, but that even mares treated 7 consecutive years did eventually return to ovulation (Kirkpatrick and Turner, 2002). Other studies have reported that continued PZP vaccine applications may result in decreased estrogen levels (Kirkpatrick et al., 1992) but that decrease was not biologically significant, as ovulation remained similar between treated and untreated mares (Powell and Monfort, 2001). Bagavant et al. (2003) demonstrated T-cell clusters on ovaries, but no loss of ovarian function after ZP protein immunization in macaques.

Reversibility and Effects on Ovaries: GnRH Vaccines
The NAS (2013) review pointed out that single doses of GonaCon-Equine do not lead to high rates of initial effectiveness, or long duration. Initial effectiveness of one dose of GonaCon-Equine vaccine appears to be lower than for a combined primer plus booster dose of the PZP vaccine Zonastat-H (Kirkpatrick et al., 2011), and the initial effect of a single GonaCon dose can be limited to as little as one breeding season. However, preliminary results on the effects of boostered doses of GonaCon-Equine indicate that it can have high efficacy and longer-lasting effects in free-roaming horses (Baker et al., 2017, 2018) than the one-year effect that is generally expected from a single booster of Zonastat-H.

Too few studies have reported on the various formulations of anti-GnRH vaccines to make generalizations about differences between products, but GonaCon formulations were consistently good at causing loss of fertility in a statistically significant fraction of treated mares for at least one year (Killian et al., 2009; Gray et al., 2010; Baker et al., 2013, 2017, 2018). With few exceptions (e.g., Goodloe, 1991), anti-GnRH treated mares gave birth to fewer foals in the first season when there would be an expected contraceptive effect (Botha et al., 2008; Killian et al., 2009; Gray et al., 2010; Baker et al., 2013, 2018). Goodloe (1991) used an anti-GnRH-KHL vaccine with a triple adjuvant, in some cases attempting to deliver the vaccine to horses with a hollow-tipped "biobullet," but concluded that the vaccine was not an effective immunocontraceptive in that study.

Not all mares should be expected to respond to the GonaCon-Equine vaccine; some number should be expected to continue to become pregnant and give birth to foals. In studies where mares were exposed to stallions, the fraction of treated mares that are effectively contracepted in the year after anti-GnRH vaccination varied from study to study, ranging from ~50% (Baker et al., 2017), to 61% (Gray et al., 2010), to ~90% (Killian et al., 2006, 2008, 2009). Miller et al. (2013) noted lower effectiveness in free-ranging mares (Gray et al. 2010) than captive mares (Killian et al., 2009). Some of these rates are lower than the high rate of effectiveness typically

reported for the first year after PZP vaccine treatment (Kirkpatrick et al., 2011). In the one study that tested for a difference, darts and hand injected GonaCon doses were equally effective in terms of fertility outcome (McCann et al., 2017).

In studies where mares were not exposed to stallions, the duration of effectiveness also varied. A primer and booster dose of Equity led to anoestrus for at least 3 months (Elhay et al., 2007). A primer and booster dose of Improvac also led to loss of ovarian cycling for all mares in the short term (Imboden et al., 2006; Nolan et al., 2018c). It is worth repeating that those vaccines do not have the same formulation as GonaCon.

Results from horses (Baker et al., 2017, 2018) and other species (Curtis et al., 2001) suggest that providing a booster dose of GonaCon-Equine will increase the fraction of temporarily infertile animals to higher levels than would a single vaccine dose alone.

Longer-term infertility has been observed in some mares treated with anti-GnRH vaccines, including GonaCon-Equine. In a single-dose mare captive trial with an initial year effectiveness of 94%, Killian et al. (2008) noted infertility rates of 64%, 57%, and 43% in treated mares during the following three years, while control mares in those years had infertility rates of 25%, 12%, and 0% in those years. GonaCon effectiveness in free-roaming populations was lower, with infertility rates consistently near 60% for three years after a single dose in one study (Gray et al., 2010) and annual infertility rates decreasing over time from 55% to 30% to 0% in another study with one dose (Baker et al., 2017, 2018). Similarly, gradually increasing fertility rates were observed after single dose treatment with GonaCon in elk (Powers et al., 2011) and deer (Gionfriddo et al., 2011a).

Baker et al. (2017, 2018) observed a return to fertility over 4 years in mares treated once with GonaCon, but then noted extremely low fertility rates of 0% and 16% in the two years after the same mares were given a booster dose four years after the primer dose. Four of nine mares treated with primer and booster doses of Improvac did not return to ovulation within 2 years of the primer dose (Imboden et al., 2006), though one should probably not make conclusions about the long-term effects of GonaCon-Equine based on results from Improvac.

It is difficult to predict which females will exhibit strong or long-term immune responses to anti-GnRH vaccines (Killian et al., 2006; Miller et al., 2008; Levy et al., 2011). A number of factors may influence responses to vaccination, including age, body condition, nutrition, prior immune responses, and genetics (Cooper and Herbert, 2001; Curtis et al., 2001; Powers et al., 2011). One apparent trend is that animals that are treated at a younger age, especially before puberty, may have stronger and longer-lasting responses (Brown et al., 1994; Curtis et al., 2001; Stout et al., 2003; Schulman et al. 2013). It is plausible that giving ConaGon-Equine to prepubertal mares will lead to long-lasting infertility, but that has not yet been tested.

To date, short term evaluation of anti-GnRH vaccines, show contraception appears to be temporary and reversible. Killian et al. noted long-term effects of GonaCon in some captive mares (2009). However, Baker et al. (2017) observed horses treated with GonaCon-B return to fertility after they were treated with a single primer dose; after four years, the fertility rate was indistinguishable between treated and control mares. It appears that a single dose of GonaCon

results in reversible infertility. If long-term treatment resulted in permanent infertility for some treated mares, such permanent infertility fertility would be consistent with the desired effect of using GonaCon (e.g., effective contraception).

Other anti-GnRH vaccines also have had reversible effects in mares. Elhay (2007) noted a return to ovary functioning over the course of 34 weeks for 10 of 16 mares treated with Equity. That study ended at 34 weeks, so it is not clear when the other six mares would have returned to fertility. Donovan et al. (2013) found that half of mares treated with an anti-GnRH vaccine intended for dogs had returned to fertility after 40 weeks, at which point the study ended. In a study of mares treated with a primer and booster dose of Improvac, 47 of 51 treated mares had returned to ovarian cyclicity within 2 years; younger mares appeared to have longer-lasting effects than older mares (Schulman et al., 2013). Joonè et al. (2017) analyzed samples from the Schulman et al. (2013) study and found no significant decrease in anti-Mullerian hormone (AMH) levels in mares treated with GnRH vaccine. AMH levels are thought to be an indicator of ovarian function, so results from Joonè et al. (2017) support the general view that the anoestrus resulting from GnRH vaccination is physiologically similar to typical winter anoestrus.

In a small study with a non-commercial anti-GnRH vaccine (Stout et al., 2003), three of seven treated mares had returned to cyclicity within 8 weeks after delivery of the primer dose, while four others were still suppressed for 12 or more weeks. In elk, Powers et al. (2011) noted that contraception after one dose of GonaCon was reversible. In white-tailed deer, single doses of GonaCon appeared to confer two years of contraception (Miller et al., 2000). Ten of 30 domestic cows treated became pregnant within 30 weeks after the first dose of Bopriva (Balet et al., 2014). Permanent sterility as a result of single-dose or boostered GonaCon-Equine vaccine, or other anti-GnRH vaccines, has not been recorded, but that may be because no long-term studies have tested for that effect. It is conceivable that some fraction of mares could become sterile after receiving one or more booster doses of GonaCon-Equine. If some fraction of mares treated with GonaCon-Equine were to become sterile, though, that result would be consistent with text of the WFRHBA, as amended, which allows for sterilization to achieve population goals.

In summary, based on the above results related to fertility effects of GonaCon and other anti-GnRH vaccines, application of a single dose of GonaCon-Equine to gathered or remotely darted wild horses could be expected to prevent pregnancy in perhaps 30%-60% of mares for one year. Some smaller number of wild mares should be expected to have persistent contraception for a second year, and less still for a third year. Applying one booster dose of GonaCon to previously treated mares may lead to four or more years with relatively high rates (80+%) of additional infertility expected (Baker et al., 2018). There is no data to support speculation regarding efficacy of multiple boosters of GonaCon-Equine; however, given it is formulated as a highly immunogenic long-lasting vaccine, it is reasonable to hypothesize that additional boosters would increase the effectiveness and duration of the vaccine.

GonaCon-Equine only affects the fertility of treated animals; untreated animals will still be expected to give birth. Even under favorable circumstances for population growth suppression, gather efficiency might not exceed 85% via helicopter, and may be less with bait and water trapping. Similarly, not all animals may be approachable for darting. The uncaptured or undarted portion of the female population would still be expected to have normally high fertility rates in

any given year, though those rates could go up slightly if contraception in other mares increases forage and water availability.

Changes in hormones associated with anti-GnRH vaccination lead to measurable changes in ovarian structure and function. The volume of ovaries reduced in response to treatment (Garza et al., 1986; Dalin et al., 2002; Imboden et al., 2006; Elhay et al., 2007; Botha et al., 2008; Gionfriddo, 2011a; Dalmau et al., 2015). Treatment with an anti-GnRH vaccine changes follicle development (Garza et al., 1986; Stout et al., 2003; Imboden et al., 2006; Elhay et al., 2007; Donovan et al. 2013, Powers et al. 2011, Balet et al. 2014), with the result that ovulation does not occur. A related result is that the ovaries can exhibit less activity and cycle with less regularity or not at all in anti-GnRH vaccine treated females (Goodloe, 1991; Dalin et al., 2002; Imboden et al., 2006; Elhay et al., 2007; Janett et al., 2009a; Powers et al., 2011; Donovan et al., 2013). In studies where the vaccine required a booster, hormonal and associated results were generally observed within several weeks after delivery of the booster dose.

Effects on Existing Pregnancies, Foals, and Birth Phenology: PZP Vaccines
Although fetuses are not explicitly protected under the WFRHBA, as amended, it is prudent to analyze the potential effects of fertility control vaccines on developing fetuses and foals. Any impacts identified in the literature have been found to be transient, and do not influence the future reproductive capacity of offspring born to treated females.

If a mare is already pregnant, the PZP vaccine has not been shown to affect normal development of the fetus or foal, or the hormonal health of the mare with relation to pregnancy (Kirkpatrick and Turner, 2003). Studies on Assateague Island (Kirkpatrick and Turner, 2002) showed that once female offspring born to mares treated with PZP during pregnancy eventually breed, they produce healthy, viable foals. It is possible that there may be transitory effects on foals born to mares or jennies treated with PZP. For example, in mice, Sacco et al. (1981) found that antibodies specific to PZP can pass from mother mouse to pup via the placenta or colostrum, but that did not apparently cause any innate immune response in the offspring: the level of those antibodies were undetectable by 116 days after birth. There was no indication in that study that the fertility or ovarian function of those mouse pups was compromised, nor is BLM aware of any such results in horses or burros. Unsubstantiated, speculative connections between PZP treatment and "foal stealing" has not been published in a peer-reviewed study and thus cannot be verified. "Foal stealing," where a near-term pregnant mare steals a neonate foal from a weaker mare, is unlikely to be a common behavioral result of including spayed mares in a wild horse herd. McDonnell (2012) noted that "foal stealing is rarely observed in horses, except under crowded conditions and synchronization of foaling," such as in horse feed lots. Those conditions are not likely in the wild, where pregnant mares will be widely distributed across the landscape, and where the expectation is that parturition dates would be distributed across the normal foaling season. Similarly, although Nettles (1997) noted reported stillbirths after PZP treatments in cynomolgus monkeys, those results have not been observed in equids despite extensive use in horses and burros.

On-range observations from 20 years of application to wild horses indicate that PZP application in wild mares does not generally cause mares to give birth to foals out of season or late in the year (Kirkpatrick and Turner, 2003). Nuñez's (2010) research showed that a small number of

mares that had previously been treated with PZP foaled later than untreated mares and expressed the concern that this late foaling "may" impact foal survivorship and decrease band stability, or that higher levels of attention from stallions on PZP-treated mares might harm those mares. However, that paper provided no evidence that such impacts on foal survival or mare well-being actually occurred. Rubenstein (1981) called attention to a number of unique ecological features of horse herds on Atlantic barrier islands, such as where Nuñez made observations, which calls into question whether inferences drawn from island herds can be applied to western wild horse herds.

Ransom et al. (2013), though, did identify a potential shift in reproductive timing as a possible drawback to prolonged treatment with PZP, stating that treated mares foaled on average 31 days later than non-treated mares. Results from Ransom et al. (2013), however, showed that over 81% of the documented births in that study were between March 1 and June 21, i.e., within the normal, peak, spring foaling season. Ransom et al. (2013) pointedly advised that managers should consider carefully before using fertility control vaccines in small refugia or rare species. Wild horses and burros managed by BLM do not generally occur in isolated refugia, nor are they at all rare species. The U.S. Fish and Wildlife Service (USFWS) denied a petition to list wild horses as endangered (USFWS 2015). Moreover, any effect of shifting birth phenology was not observed uniformly: in two of three PZP-treated wild horse populations studied by Ransom et al. (2013), foaling season of treated mares extended three weeks and 3.5 months, respectively, beyond that of untreated mares. In the other population, the treated mares foaled within the same time period as the untreated mares. Furthermore, Ransom et al. (2013) found no negative impacts on foal survival even with an extended birthing season. If there are shifts in birth phenology, though, it is reasonable to assume that some negative effects on foal survival for a small number of foals might result from particularly severe weather events (Nuñez et al., 2018).

Effects on Existing Pregnancies, Foals, and Birth Phenology: GnRH Vaccines
As stated in the previous section, although fetuses are not explicitly protected under the WFRHBA, as amended, it is prudent to analyze the potential effects of fertility control vaccines on developing fetuses and foals. Any impacts identified in the literature have been found to be transient, and do not influence the future reproductive capacity of offspring born to treated females.

GonaCon and other anti-GnRH vaccines can be injected while a female is pregnant (Miller et al., 2000; Powers et al., 2011; Baker et al., 2013) – in such a case, a successfully contracepted mare will be expected to give birth during the following foaling season, but to be infertile during the same year's breeding season. Thus, a mare injected in November of 2018 would not show the contraceptive effect (i.e., no new foal) until spring of 2020.

GonaCon had no apparent effect on pregnancies in progress, foaling success, or the health of offspring, in horses that were immunized in October (Baker et al., 2013), elk immunized 80-100 days into gestation (Powers et al., 2011, 2013), or deer immunized in February (Miller et al., 2000). Kirkpatrick et al. (2011) noted that anti-GnRH immunization is not expected to cause hormonal changes that would lead to abortion in the horse, but this may not be true for the first 6 weeks of pregnancy (NAS, 2013). Curtis et al. (2011) noted that GonaCon-KHL treated white tailed deer had lower twinning rates than controls but speculated that the difference could be due

to poorer sperm quality late in the breeding season, when the treated does did become pregnant. Goodloe (1991) found no difference in foal production between treated and control animals.

Offspring of anti-GnRH vaccine treated mothers could exhibit an immune response to GnRH (Khodr and Siler-Khodr, 1980), as antibodies from the mother could pass to the offspring through the placenta or colostrum. In the most extensive study of long-term effects of GonaCon immunization on offspring, Powers et al. (2012) monitored 15 elk fawns born to GonaCon treated cows. Of those, 5 had low titers at birth and 10 had high titer levels at birth. All 15 were of normal weight at birth, and developed normal endocrine profiles, hypothalamic GnRH content, pituitary gonadotropin content, gonad structure, and gametogenesis. All the females became pregnant in their second reproductive season, as is typical. All males showed normal development of secondary sexual characteristics. Powers et al. (2012) concluded that suppressing GnRH in the neonatal period did not alter long-term reproductive function in either male or female offspring. Miller et al. (2013) report elevated anti-GnRH antibody titers in fawns born to treated white tailed deer, but those dropped to normal levels in 11 of 12 of those fawns, which came into breeding condition; the remaining fawn was infertile for three years.

Direct effects on foal survival are equivocal in the literature. Goodloe (1991) reported lower foal survival for a small sample of foals born to anti-GnRH treated mares, but she did not assess other possible explanatory factors such as mare social status, age, body condition, or habitat in her analysis (NAS, 2013). Gray et al. (2010) found no difference in foal survival in foals born to free-roaming mares treated with GonaCon.

There is little empirical information available to evaluate the effects of GnRH vaccination on foaling phenology, but those effects are likely to be similar to those for PZP vaccine treated mares in which the effects of the vaccine wear off. It is possible that immunocontracepted mares returning to fertility late in the breeding season could give birth to foals at a time that is out of the normal range (Nuñez et al., 2010; Ransom et al., 2013). Curtis et al. (2001) did observe a slightly later fawning date for GonaCon treated deer in the second year after treatment, when some does regained fertility late in the breeding season. In anti-GnRH vaccine trials in free-roaming horses, there were no published differences in mean date of foal production (Goodloe, 1991; Gray et al., 2010). Unpublished results from an ongoing study of GonaCon treated free-roaming mares indicate that some degree of seasonal foaling is possible (D. Baker, Colorado State University, personal communication to Paul Griffin, BLM Wild Horse and Burro Research Coordinator).

Because of the concern that contraception could lead to shifts in the timing of parturitions for some treated animals, Ransom et al. (2013) advised that managers should consider carefully before using PZP immunocontraception in small refugia or rare species; the same considerations could be advised for use of GonaCon, but wild horses and burros in most areas do not generally occur in isolated refugia, they are not a rare species at the regional, national, or international level, and genetically they represent descendants of domestic livestock with most populations containing few if any unique alleles (NAS, 2013). Moreover, in PZP-treated horses that did have some degree of parturition date shift, Ransom et al. (2013) found no negative impacts on foal survival even with an extended birthing season; however, this may be more related to stochastic, inclement weather events than extended foaling seasons. If there were to be a shift in foaling date

for some treated mares, the effect on foal survival may depend on weather severity and local conditions; for example, Ransom et al. (2013) did not find consistent effects across study sites.

Effects of Marking and Injection
Standard practices require that immunocontraceptive-treated animals be readily identifiable, either via brand marks or unique coloration (BLM, 2010). Some level of transient stress is likely to result in newly captured mares that do not have markings associated with previous fertility control treatments. It is difficult to compare that level of temporary stress with the long-term stress that can result from food and water limitation on the range (e.g., Creel et al., 2013). Handling may include freeze-marking, for the purpose of identifying that mare and identifying her vaccine treatment history. Under past management practices, captured mares experienced increased stress levels from handling (Ashley and Holcombe, 2001), but BLM has instituted guidelines to reduce the sources of handling stress in captured animals (BLM, 2015).

Most mares recover from the stress of capture and handling quickly once released back to the range, and none are expected to suffer serious long-term effects from the fertility control injections, other than the direct consequence of becoming temporarily infertile. Injection site reactions associated with fertility control treatments are possible in treated mares (Roelle and Ransom, 2009; Bechert et al., 2013; French et al., 2017; Baker et al., 2018), but swelling or local reactions at the injection site are expected to be minor in nature. Roelle and Ransom (2009) found that the most time-efficient method for applying PZP is by hand-delivered injection of 2-year pellets when horses are gathered. They observed only two instances of swelling from that technique. Whether injection is by hand or via darting, GonaCon-Equine is associated with some degree of inflammation, swelling, and the potential for abscesses at the injection site (Baker et al., 2013). Swelling or local reactions at the injection site are generally expected to be minor in nature, but some may develop into draining abscesses. Use of remotely delivered vaccine is generally limited to populations where individual animals can be accurately identified and repeatedly approached. The dart-delivered PZP formulation produced injection-site reactions of varying intensity, though none of the observed reactions appeared debilitating to the animals (Roelle and Ransom, 2009) but that was not observed with dart-delivered GonaCon (McCann et al., 2017). Joonè et al. (2017a) found that injection site reactions had healed in most mares within 3 months after the booster dose, and that they did not affect movement or cause fever.

Long-lasting nodules observed did not appear to change any animal's range of movement or locomotor patterns and in most cases did not appear to differ in magnitude from naturally occurring injuries or scars. Mares treated with one formulation of GnRH-KHL vaccine developed pyogenic abscesses (Goodloe, 1991). Miller et al. (2008) noted that the water and oil emulsion in GonaCon will often cause cysts, granulomas, or sterile abscesses at injection sites; in some cases, a sterile abscess may develop into a draining abscess. In elk treated with GonaCon, Powers et al. (2011) noted up to 35% of treated elk had an abscess form, despite the injection sites first being clipped and swabbed with alcohol. Even in studies where swelling and visible abscesses followed GonaCon immunization, the longer-term nodules observed did not appear to change any animal's range of movement or locomotor patterns (Powers et al., 2013; Baker et al., 2017, 2018).

The result that other formulations of anti-GnRH vaccine may be associated with less notable injection site reactions in horses may indicate that the adjuvant formulation in GonaCon leads a single dose to cause a stronger immune reaction than the adjuvants used in other anti-GnRH vaccines. Despite that, a booster dose of GonaCon-Equine appears to be more effective than a primer dose alone (Baker et al., 2017). Horses injected in the hip with Improvac showed only transient reactions that disappeared within 6 days in one study (Botha et al., 2008), but stiffness and swelling that lasted 5 days were noted in another study where horses received Improvac in the neck (Imboden et al., 2006). Equity led to transient reactions that resolved within a week in some treated animals (Elhay et al., 2007). Donovan et al. noted no reactions to the canine anti-GnRH vaccine (2013). In cows treated with Bopriva there was a mildly elevated body temperature and mild swelling at injection sites that subsided within 2 weeks (Balet et al., 2014).

Indirect Effects: PZP Vaccines
One expected long-term, indirect effect on wild horses treated with fertility control would be an improvement in their overall health (Turner and Kirkpatrick, 2002). Many treated mares would not experience the biological stress of reproduction, foaling, and lactation as frequently as untreated mares. The observable measure of improved health is higher body condition scores (Nuñez et al., 2010). After a treated mare returns to fertility, her future foals would be expected to be healthier overall and would benefit from improved nutritional quality in the mare's milk. This is particularly to be expected if there is an improvement in rangeland forage quality at the same time, due to reduced wild horse population size. Past application of fertility control has shown that mares' overall health and body condition remains improved even after fertility resumes. PZP treatment may increase mare survival rates, leading to longer potential lifespan (Turner and Kirkpatrick, 2002; Ransom et al., 2014a) that may be as much as 5-10 years (NPS, 2008). To the extent that this happens, changes in lifespan and decreased foaling rates could combine to cause changes in overall age structure in a treated herd (i.e., Turner and Kirkpatrick, 2002; Roelle et al., 2010), with a greater prevalence of older mares in the herd (Gross, 2000; NPS, 2008). Observations of mares treated in past gathers showed that many of the treated mares were larger than, maintained higher body condition than, and had larger healthy foals than untreated mares (BLM, anecdotal observations).

Following resumption of fertility, the proportion of mares that conceive and foal could be increased due to their increased fitness; this has been called a "rebound effect." Elevated fertility rates have been observed after horse gathers and removals (Kirkpatrick and Turner, 1991). If repeated contraceptive treatment leads to a prolonged contraceptive effect, then that may minimize or delay the hypothesized rebound effect. Selectively applying contraception to older animals and returning them to the range could reduce long-term holding costs for such horses, which are difficult to adopt, and may reduce the compensatory reproduction that often follows removals (Kirkpatrick and Turner, 1991).

Because successful fertility control in a given herd reduces foaling rates and population growth rates, another indirect effect should be to reduce the number of wild horses that have to be removed over time to achieve and maintain the established AML. Contraception may change a herd's age structure, with a relative increase in the fraction of older animals in the herd (NPS, 2008). Reducing the numbers of wild horses that would have to be removed in future gathers could allow for removal of younger, more easily adoptable excess wild horses, and thereby could

eliminate the need to send additional excess horses from this area to off-range holding corrals or pastures for long-term holding.

A principal motivation for use of contraceptive vaccines or sex ratio adjustment is to reduce population growth rates and maintain herd sizes at AML. Where successful, this should allow for continued and increased environmental improvements to range conditions within the project area, which would have long-term benefits to wild horse and burro habitat quality, and well-being of animals living on the range. As the population nears or is maintained at the level necessary to achieve a thriving natural ecological balance, vegetation resources would be expected to recover, improving the forage available. With rangeland conditions more closely approaching a thriving natural ecological balance, and with a less concentrated distribution of wild horses and burros, there should also be less trailing and concentrated use of water sources. Lower population density should lead to reduced competition among wild horses using the water sources, and less fighting among horses accessing water sources. Water quality and quantity would continue to improve to the benefit of all rangeland users including wild horses. Wild horses would also have to travel less distance back and forth between water and desirable foraging areas. Among mares in the herd that remain fertile, a higher level of physical health and future reproductive success would be expected in areas where lower horse and burro population sizes lead to increases in water and forage resources. While it is conceivable that widespread and continued treatment with fertility control vaccines could reduce the birth rates of the population to such a point that birth is consistently below mortality, that outcome is not likely unless a very high fraction of the mares present are treated in almost every year.

Indirect Effects: GnRH Vaccines
As noted above with respect to PZP vaccines, an expected long-term, indirect effect on wild horses treated with fertility control would be an improvement in their overall health. Body condition of anti-GnRH-treated females was equal to or better than that of control females in published studies. Ransom et al. (2014b) observed no difference in mean body condition between GonaCon-B treated mares and controls. Goodloe (1991) found that GnRH-KHL treated mares had higher survival rates than untreated controls. In other species, treated deer had better body condition than controls (Gionfriddo et al., 2011b), treated cats gained more weight than controls (Levy et al. 2011), as did treated young female pigs (Bohrer et al., 2014).

Following resumption of fertility, the proportion of mares that conceive and foal could be increased due to their increased fitness; this has been called by some a "rebound effect." Elevated fertility rates have been observed after horse gathers and removals (Kirkpatrick and Turner, 1991). If repeated contraceptive treatment leads to a prolonged contraceptive effect, then that may minimize or delay the hypothesized rebound effect. Selectively applying contraception to older animals and returning them to the range could reduce long-term holding costs for such horses, which are difficult to adopt, and could negate the compensatory reproduction that can follow removals (Kirkpatrick and Turner, 1991).

Because successful fertility control would reduce foaling rates and population growth rates, another indirect effect would be to reduce the number of wild horses that have to be removed over time to achieve and maintain the established AML. Contraception would be expected to lead to a relative increase in the fraction of older animals in the herd. Reducing the numbers of

wild horses that would have to be removed in future gathers could allow for removal of younger, more easily adoptable excess wild horses, and thereby could eliminate the need to send additional excess horses from this area to off-range holding corrals or pastures for long-term holding. Among mares in the herd that remain fertile, a high level of physical health and future reproductive success would be expected because reduced population sizes should lead to more availability of water and forage resources per capita.

Reduced population growth rates and smaller population sizes could also allow for continued and increased environmental improvements to range conditions within the project area, which would have long-term benefits to wild horse habitat quality. As the local horse abundance nears or is maintained at the level necessary to achieve a thriving natural ecological balance, vegetation resources would be expected to recover, improving the forage available to wild horses and wildlife throughout the area. With rangeland conditions more closely approaching a thriving natural ecological balance, and with a less concentrated distribution of wild horses across the range, there should also be less trailing and concentrated use of water sources. Lower population density would be expected to lead to reduced competition among wild horses using the water sources, and less fighting among horses accessing water sources. Water quality and quantity would continue to improve to the benefit of all rangeland users including wild horses. Wild horses would also have to travel less distance back and forth between water and desirable foraging areas. Should GonaCon-Equine treatment, including booster doses, continue into the future, with treatments given on a schedule to maintain a lowered level of fertility in the herd, the chronic cycle of overpopulation and large gathers and removals might no longer occur, but instead a consistent abundance of wild horses could be maintained, resulting in continued improvement of overall habitat conditions and animal health. While it is conceivable that widespread and continued treatment with GonaCon-Equine could reduce the birth rates of the population to such a point that birth is consistently below mortality, that outcome is not likely unless a very high fraction of the mares present are all treated with primer and booster doses, and perhaps repeated booster doses.

Behavioral Effects: PZP Vaccines
Behavioral difference, compared to mares that are fertile, should be considered as potential results of successful contraception. The NAS report (2013) noted that all forms of fertility suppression have effects on mare behavior, mostly because of the lack of pregnancy and foaling, and concluded that fertility control vaccines were among the most promising fertility control methods for wild horses and burros. The resulting impacts may be seen as neutral in the sense that a wide range of natural behaviors is already observable in untreated wild horses, or mildly adverse in the sense that effects are expected to be transient and to not affect all treated animals.

PZP vaccine-treated mares may continue estrus cycles throughout the breeding season. Ransom and Cade (2009) delineated wild horse behaviors. Ransom et al. (2010) found no differences in how PZP-treated and untreated mares allocated their time between feeding, resting, travel, maintenance, and most social behaviors in three populations of wild horses, which is consistent with Powell's (1999) findings in another population. Likewise, body condition of PZP-treated and control mares did not differ between treatment groups in Ransom et al.'s (2010) study. Nuñez (2010) found that PZP-treated mares had higher body condition than control mares in another population, presumably because energy expenditure was reduced by the absence of

pregnancy and lactation. Knight (2014) found that PZP-treated mares had better body condition, lived longer and switched harems more frequently, while mares that foaled spent more time concentrating on grazing and lactation and had lower overall body condition.

In two studies involving a total of four wild horse populations, both Nuñez et al. (2009) and Ransom et al. (2010) found that PZP vaccine treated mares were involved in reproductive interactions with stallions more often than control mares, which is not surprising given the evidence that PZP-treated females of other mammal species can regularly demonstrate estrus behavior while contracepted (Shumake and Killian, 1997; Heilmann et al., 1998; Curtis et al., 2001; Duncan et al., 2017). There was no evidence, though, that mare welfare was affected by the increased level of herding by stallions noted in Ransom et al. (2010). Nuñez's later analysis (2017) noted no difference in mare reproductive behavior as a function of contraception history.

Ransom et al. (2010) found that control mares were herded by stallions more frequently than PZP-treated mares, and Nuñez et al. (2009, 2014, 2017, 2018) found that PZP-treated mares exhibited higher infidelity to their band stallion during the non-breeding season than control mares. Madosky et al. (2010) and Knight (2014) found this infidelity was also evident during the breeding season in the same population that Nuñez et al. (2009, 2010, 2014, 2017, 2018) studied. Nuñez et al. (2014, 2017, 2018) concluded that PZP-treated mares changing bands more frequently than control mares could lead to band instability. Nuñez et al. (2009), though, cautioned against generalizing from that island population to other herds. Also, despite any potential changes in band infidelity due to PZP vaccination, horses continued to live in social groups with dominant stallions and one or more mares. Nuñez et al. (2014) found elevated levels of fecal cortisol, a marker of physiological stress, in mares that changed bands. The research is inconclusive as to whether all the mares' movements between bands were related to the PZP treatments themselves or the fact that the mares were not nursing a foal and did not demonstrate any long-term negative consequence of the transiently elevated cortisol levels.

In separate work in a long-term study of semi-feral Konik ponies, Jaworska et al. (2020) showed that neither infanticide nor feticide resulted for mares and their foals after a change in dominant stallion. Nuñez et al. 2014 wrote that these effects "…may be of limited concern when population reduction is an urgent priority." Nuñez (2018) and Jones et al. (2019, 2020) noted that band stallions of mares that have received PZP treatment can exhibit changes in behavior and physiology. Nuñez (2018) cautioned that PZP use may limit the ability of mares to return to fertility, but also noted that, "such aggressive treatments may be necessary when rapid reductions in animal numbers are of paramount importance…If the primary management goal is to reduce population size, it is unlikely (and perhaps less important) that managers achieve a balance between population control and the maintenance of more typical feral horse behavior and physiology."

In contrast to transient stresses, Creel et al. (2013) highlight that variation in population density is one of the most well-established causal factors of chronic activation of the hypothalamic-pituitary-adrenal axis, which mediates stress hormones; high population densities and competition for resources can cause chronic stress. Creel et al. (2013) also state that "…there is little consistent evidence for a negative association between elevated baseline glucocorticoids and fitness." Band fidelity is not an aspect of wild horse biology that is specifically protected by

the WFRHBA. It is also notable that Ransom et al. (2014b) found higher group fidelity after a herd had been gathered and treated with a contraceptive vaccine; in that case, the researchers postulated that higher fidelity may have been facilitated by the decreased competition for forage after excess horses were removed. At the population level, available research does not provide evidence of the loss of harem structure among any herds treated with PZP. No biologically significant negative impacts on the overall animals or populations overall, long-term welfare or well-being have been established in these studies.

The NAS (2013) found that harem changing was not likely to result in serious adverse effects for treated mares:

> "The studies on Shackleford Banks (Nuñez et al., 2009; Madosky et al., 2010) suggest that there is an interaction between pregnancy and social cohesion. The importance of harem stability to mare well-being is not clear but considering the relatively large number of free-ranging mares that have been treated with liquid PZP in a variety of ecological settings, the likelihood of serious adverse effects seem low."

Nuñez (2010) stated that not all populations will respond similarly to PZP treatment. Differences in habitat, resource availability, and demography among conspecific populations will undoubtedly affect their physiological and behavioral responses to PZP contraception and may be considered. Kirkpatrick et al. (2010) concluded that: "the larger question is, even if subtle alterations in behavior may occur, this is still far better than the alternative," and that the "…other victory for horses is that every mare prevented from being removed, by virtue of contraception, is a mare that will only be delaying her reproduction rather than being eliminated permanently from the range. This preserves herd genetics, while gathers and adoption do not."

The NAS report (2013) provides a comprehensive review of the literature on the behavioral effects of contraception that puts research up to that date by Nuñez et al. (2009, 2010) into the broader context of all of the available scientific literature, and cautions, based on its extensive review of the literature that:

> ". . . in no case can the committee conclude from the published research that the behavior differences observed are due to a particular compound rather than to the fact that treated animals had no offspring during the study. That must be borne in mind particularly in interpreting long-term impacts of contraception (e.g., repeated years of reproductive "failure" due to contraception)."

Behavioral Effects: GnRH Vaccines
The result that GonaCon treated mares may have suppressed estrous cycles throughout the breeding season can lead treated mares to behave in ways that are functionally similar to pregnant mares. Where it is successful in mares, GonaCon and other anti-GnRH vaccines are expected to induce fewer estrous cycles when compared to non-pregnant control mares. This has been observed in many studies (Garza et al., 1986; Curtis et al., 2001; Dalin et al., 2002; Killian et al., 2006; Dalmau et al., 2015). Females treated with GonaCon had fewer estrous cycles than control or PZP-treated mares (Killian et al., 2006) or deer (Curtis et al., 2001). Thus, any concerns about PZP treated mares receiving more courting and breeding behaviors from stallions

(Nuñez et al., 2009; Ransom et al., 2010) are not generally expected to be a concern for mares treated with anti-GnRH vaccines (Botha et al., 2008).

Ransom et al. (2014b) and Baker et al. (2018) found that GonaCon treated mares had similar rates of reproductive behaviors that were similar to those of pregnant mares. Among other potential causes, the reduction in progesterone levels in treated females may lead to a reduction in behaviors associated with reproduction. Despite this, some females treated with GonaCon or other anti-GnRH vaccines did continue to exhibit reproductive behaviors, albeit at irregular intervals and durations (Dalin et al., 2002; Stout et al., 2003; Imboden et al., 2006), which is a result that is similar to spayed (ovariectomized) mares (Asa et al. 1980). Gray et al. (2009a) and Baker et al. (2018) found no difference in sexual behaviors in mares treated with GonaCon and untreated mares. When progesterone levels are low, small changes in estradiol concentration can foster reproductive estrous behaviors (Imboden et al., 2006). Owners of anti-GnRH vaccine treated mares reported a reduced number of estrous-related behaviors under saddle (Donovan et al., 2013). Treated mares may refrain from reproductive behavior even after ovaries return to cyclicity (Elhay et al., 2007). Studies in elk found that GonaCon treated cows had equal levels of precopulatory behaviors as controls (Powers et al., 2011), though bull elk paid more attention to treated cows late in the breeding season, after control cows were already pregnant (Powers et al., 2011).

Stallion herding of mares, and harem switching by mares are two behaviors related to reproduction that might change as a result of contraception. Ransom et al. (2014b) observed a 50% decrease in herding behavior by stallions after the free-roaming horse population at Theodore Roosevelt National Park was reduced via a gather, and mares there were treated with GonaCon-B. The increased harem tending behaviors by stallions were directed to both treated and control mores. It is difficult to separate any effect of GonaCon in this study from changes in horse density and forage following horse removals.

With respect to treatment with GonaCon or other anti-GnRH vaccines, it is probably less likely that treated mares will switch harems at higher rates than untreated animals, because treated mares are similar to pregnant mares in their behaviors (Ransom et al., 2014b). Indeed, Gray et al. (2009a) found no difference in band fidelity in a free-roaming population of horses with GonaCon treated mares, despite differences in foal production between treated and untreated mares. Ransom et al. (2014b) actually found increased levels of band fidelity after treatment, though this may have been partially a result of changes in overall horse density and forage availability.

Gray et al. (2009) and Ransom et al. (2014b) monitored non-reproductive behaviors in GonaCon treated populations of free-roaming horses. Gray et al. (2009a) found no difference between treated and untreated mares in terms of activity budget, sexual behavior, proximity of mares to stallions, or aggression. Ransom et al. (2014b) found only minimal differences between treated and untreated mare time budgets, but those differences were consistent with differences in the metabolic demands of pregnancy and lactation in untreated mares, as opposed to non-pregnant treated mares.

Genetic Effects of Fertility Control Vaccines
In HMAs where large numbers of wild horses have recent and/or an ongoing influx of breeding animals from other areas with wild or feral horses, contraception is not expected to cause an unacceptable loss of genetic diversity or an unacceptable increase in the inbreeding coefficient. In any diploid population, the loss of genetic diversity through inbreeding or drift can be prevented by large effective breeding population sizes (Wright, 1931) or by introducing new potential breeding animals (Mills and Allendorf, 1996). The NAS report (2013) recommended that single HMAs should not be considered as isolated genetic populations. Rather, managed herds of wild horses should be considered as components of interacting metapopulations, with the potential for interchange of individuals and genes taking place as a result of both natural and human-facilitated movements. Introducing 1-2 mares every generation (about every 10 years) is a standard management technique that can alleviated potential inbreeding concerns (BLM, 2010).

In the last 10 years, there has been a high realized growth rate of wild horses in most areas administered by the BLM, such that most alleles that are present in any given mare are likely to already be well represented in her siblings, cousins, and more distant relatives. With the exception of horses in a small number of well-known HMAs that contain a relatively high fraction of alleles associated with old Spanish horse breeds (NAS, 2013), the genetic composition of wild horses in lands administered by the BLM is consistent with admixtures from domestic breeds. As a result, in most HMAs, applying fertility control to a subset of mares is not expected to cause irreparable loss of genetic diversity. Improved longevity and an aging population are expected results of contraceptive treatment that can provide for lengthening generation time; this result would be expected to slow the rate of genetic diversity loss (Hailer et al., 2006). Based on a population model, Gross (2000) found that a strategy to preferentially treat young animals with a contraceptive led to more genetic diversity being retained than either a strategy that preferentially treats older animals, or a strategy with periodic gathers and removals.

Even if it is the case that repeated treatment with a fertility control vaccine may lead to prolonged infertility, or even sterility in some mares, most HMAs have only a low risk of loss of genetic diversity if logistically realistic rates of contraception are applied to mares. Wild horses in most herd management areas are descendants of a diverse range of ancestors coming from many breeds of domestic horses. As such, the existing genetic diversity in the majority of HMAs does not contain unique or historically unusual genetic markers. Past interchange between HMAs, either through natural dispersal or through assisted migration (i.e., human movement of horses) means that many HMAs are effectively indistinguishable and interchangeable in terms of their genetic composition (i.e., see the table of Fst vales *in* NAS [2013]). Roelle and Oyler-McCance (2015) used the VORTEX population model to simulate how different rates of mare sterility would influence population persistence and genetic diversity, in populations with high or low starting levels of genetic diversity, various starting population sizes, and various annual population growth rates.

Their results show that the risk of the loss of genetic heterozygosity is extremely low except in case where all of the following conditions are met: starting levels of genetic diversity are low, initial population size is 100 or less, the intrinsic population growth rate is low (5% per year), and very large fractions of the female population are permanently sterilized.

It is worth noting that, although maintenance of genetic diversity at the scale of the overall population of wild horses is an intuitive management goal, there are no existing laws or policies that require BLM to maintain genetic diversity at the scale of the individual herd management area or complex. Also, there is no Bureau-wide policy that requires BLM to allow each female in a herd to reproduce before she is treated with contraceptives.

One concern that has been raised with regards to genetic diversity is that treatment with immunocontraceptives could possibly lead to an evolutionary increase in the frequency of individuals whose genetic composition fosters weak immune responses (Cooper and Larson, 2006; Ransom et al., 2014a).Many factors influence the strength of a vaccinated individual's immune response, potentially including genetics, but also nutrition, body condition, and prior immune responses to pathogens or other antigens (Powers et al., 2013). This premise is based on an assumption that lack of response to any given fertility control vaccine is a heritable trait, and that the frequency of that trait will increase over time in a population of vaccine-treated animals. Cooper and Herbert (2001) reviewed the topic, in the context of concerns about the long-term effectiveness of immunocontraceptives as a control agent for exotic species in Australia. They argue that imunocontraception could be a strong selective pressure, and that selecting for reproduction in individuals with poor immune response could lead to a general decline in immune function in populations where such evolution takes place. Other authors have also speculated that differences in antibody titer responses could be partially due to genetic differences between animals (Curtis et al., 2001; Herbert and Trigg, 2005). However, Magiafolou et al. (2013) clarify that if the variation in immune response is due to environmental factors (i.e., body condition, social rank) and not due to genetic factors, then there will be no expected effect of the immune phenotype on future generations. It is possible that general health, as measured by body condition, can have a causal role in determining immune response, with animals in poor condition demonstrating poor immune reactions (NAS, 2013).

Correlations between physical factors and immune response would not preclude, though, that there could also be a heritable response to immunocontraception. In studies not directly related to immunocontraception, immune response has been shown to be heritable (Kean et al., 1994; Sarker et al., 1999). Unfortunately, predictions about the long-term, population-level evolutionary response to immunocontraceptive treatments are speculative at this point, with results likely to depend on several factors, including: the strength of the genetic predisposition to not respond to the fertility control vaccine; the heritability of that gene or genes; the initial prevalence of that gene or genes; the number of mares treated with a primer dose of the vaccine (which generally has a short-acting effect); the number of mares treated with one or more booster doses of the vaccine; and the actual size of the genetically-interacting metapopulation of horses within which the vaccine treatment takes place.

BLM is not aware of any studies that have quantified the heritability of a lack of response to immunocontraception such as PZP vaccine or GonaCon-Equine in horses or burros. At this point, there are no studies available from which one could make conclusions about the long-term effects of sustained and widespread immunocontraception treatments on population-wide immune function. Although a few, generally isolated, feral horse populations have been treated with high fractions of mares receiving PZP immunocontraception for long-term population

control (e.g., Assateague Island National Park, and Pryor Mountains Herd Management Area), no studies have tested for changes in immune competence in those areas. Relative to the large number of free-roaming feral horses in the western United States, immunocontraception has not been, and is not expected to be used in the type of widespread or prolonged manner that might be required to cause a detectable evolutionary response.

Sex Ratio Adjustment
Skewing the sex ratio of a herd so that there are more males than females is an established BLM management technique for reducing population growth rates. As part of a wild horse and burro gather process, the number of animals returned to the range may include more males, the number removed from the range may include more females, or both. By reducing the proportion of breeding females in a population (as a fraction of the total number of animals present), the technique leads to fewer foals being born, relative to the total herd size.

Sex ratio is typically adjusted in such a way that 60 percent of the horses are male. In the absence of other fertility control treatments, this 60:40 sex ratio can temporarily reduce population growth rates from approximately 20% to approximately 15% (Bartholow, 2004). While such a decrease in growth rate may not appear to be large or long-lasting, the net result can be that fewer foals being born, at least for a few years – this can extend the time between gathers, and reduce impacts on-range, and costs off-range. Any impacts of sex ratio adjustment are expected to be temporary because the sex ratio of wild horse and burro foals at birth is approximately equal between males and females (NAS, 2013), and it is common for female foals to reproduce by their second year (NAS, 2013). Thus, within a few years after a gather and selective removal that leads to more males than females, the sex ratio of reproducing wild horses and burros will be returning toward a 50:50 ratio.

Having a larger number of males than females is expected to lead to several demographic and behavioral changes as noted in the NAS report (2013), including the following. Having more fertile males than females should not alter the fecundity of fertile females. Wild mares may be distributed in a larger number of smaller harems. Competition and aggression between males may cause a decline in male body condition. Female foraging may be somewhat disrupted by elevated male-male aggression. With a greater number of males available to choose from, females may have opportunities to select more genetically fit sires. There would also be an increase the genetic effective population size because more stallions would be breeding, and existing females would be distributed among many more small harems. This last beneficial impact is one reason that skewing the sex ratio to favor males is listed in the BLM wild horse and burro handbook (BLM 2010) as a method to consider in herds where there may be concern about the loss of genetic diversity; having more males fosters a greater retention of genetic diversity.

Infanticide is a natural behavior that has been observed in wild equids (Feh and Munktuya, 2008; Gray, 2009), but there are no published accounts of infanticide rates increasing as a result of having a skewed sex ratio in wild horse or wild burro herds. Any comment that implies such an impact would be speculative.

The BLM wild horse and burro management handbook (BLM, 2010) discusses this method. The handbook acknowledges that there may be some behavioral impacts of having more males than females. The handbook includes guidelines for when the method should be applied, specifying that this method should be considered where the low end of the AML is 150 animals or greater, and with the result that males comprise 60-70 percent of the herd. Having more than 70 percent males may result in unacceptable impacts in terms of elevated male-male aggression. In NEPA analyses, BLM has chosen to follow these guidelines in some cases, for example:

- In the 2015 Cold Springs HMA Population Management Plan EA (DOI-BLM-V040-2015-022), the low end of AML was 75. Under the preferred alternative, 37 mares and 38 stallions would remain on the HMA. This is well below the 150 head threshold noted above.
- In the 2017 Hog Creek HMA Population Management Plan EA (DOI-BLM-ORWA-V000-2017-0026-EA), BLM clearly identified that maintaining a 50:50 sex ratio was appropriate because the herd size at the low end of AML was only 30 animals.

It is relatively straightforward to speed the return of skewed sex ratios back to a 50:50 ratio. The BLM wild horse and burro handbook (BLM 2010) specifies that, if post-treatment monitoring reveals negative impacts to breeding harems due to sex ratio adjustment, then mitigation measures could include removing males, not introducing additional males, or releasing a larger proportion of females during the next gather.

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Effects of Intrauterine Devices (IUDs)

Based on promising results from published, peer-reviewed studies in domestic mares, BLM has begun to use IUDs to control fertility as a wild horse and burro fertility control method on the range. The initial management use was in mares from the Swasey HMA, in Utah. The BLM has supported and continues to support research into the development and testing of effective and safe IUDs for use in wild horse mares (Baldrighi et al., 2017; Holyoak et al., 2021). However, existing literature on the use of IUDs in horses allows for inferences about expected effects of any management alternatives that might include use of IUDs and support the apparent safety and efficacy of some types of IUDs for use in horses. Overall, as with other methods of population growth suppression, use of IUDs and other fertility control measures are expected to help reduce population growth rates, extend the time interval between gathers, and reduce the total number of excess animals that will need to be removed from the range.

The 2013 National Research Council of the National Academy of Sciences (NAS) report considered IUDs and suggested that research should test whether IUDs cause uterine inflammation and should also test how well IUDs stay in mares that live and breed with fertile stallions. Since that report, a recent study by Holyoak et al. (2021) indicate that a flexible, inert, Y-shaped, medical-grade silicone IUD design prevented pregnancies in all the domestic mares that retained the device, even when exposed to fertile stallions. Domestic mares in that study lived in large pastures, mating with fertile stallions. Biweekly ultrasound examinations showed that IUDs stayed in 75% of treated mares over the course of two breeding seasons. The IUDs were then removed so the researchers could monitor the mares' return to fertility. In that study, uterine health, as measured in terms of inflammation, was not seriously affected by the IUDs, and most mares became pregnant within months after IUD removal. The overall results are consistent with results from an earlier study (Daels and Hughes, 1995), which used O-shaped silicone IUDs. Similarly, a flexible IUD with three components connected by magnetic force (the 'iUPOD') was retained over 90 days in mares living and breeding with a fertile stallion; after IUD removal, the majority of mares became pregnant in the following breeding season (Hoopes et al., 2021).

IUDs are considered a temporary fertility control method that does not generally cause future sterility (Daels and Hughes, 1995). Use of IUDs is an effective fertility control method in women, and IUDs have historically been used in livestock management, including in domestic horses. Insertion of an IUD can be a very rapid procedure, but it does require the mare to be temporarily restrained, such as in a squeeze chute. IUDs in mares may cause physiological effects including discomfort, infection, perforation of the uterus if the IUD is hard and angular, endometritis, uterine edema (Killian et al., 2008), and pyometra (Klabnik-Bradford et al., 2013). In women, deaths attributable to IUD use may be as low as 1.06 per million (Daels and Hughes, 1995). The effects of IUD use on genetic diversity in a given herd should be comparable to those of other temporary fertility control methods; use should reduce the fraction of mares breeding at

any one time but does not necessarily preclude treated mares from breeding in the future, as they survive and regain fertility.

The exact mechanism by which IUDs prevent pregnancy is uncertain, but may be related to persistent, low-grade uterine inflammation (Daels and Hughes, 1995; Gradil et al., 2021; Hoopes et al., 2021), Turner et al. (2015) suggested that the presence of an IUD in the uterus may, like a pregnancy, prevent the mare from coming back into estrus. However, some domestic mares did exhibit repeated estrus cycles during the time when they had IUDs (Killian et al., 2008; Gradil et al., 2019; Lyman et al., 2021; Hoopes et al., 2021). The main cause for an IUD to not be effective at contraception is its failure to stay in the uterus (Daels and Hughes, 1995; NAS, 2013). As a result, one of the major challenges to using IUDs to control fertility in mares on the range is preventing the IUD from being dislodged or otherwise ejected over the course of daily activities, which could include, at times, frequent breeding.

At this time, it is thought that any IUD inserted into a pregnant mare may cause the pregnancy to terminate, which may also cause the IUD to be expelled. For that reason, it is expected that IUDs would only be inserted in non-pregnant (open) mares. Wild mares receiving IUDs would be checked for pregnancy by a veterinarian prior to insertion of an IUD. This can be accomplished by transrectal palpation and/or ultrasound performed by a veterinarian. Pregnant mares would not receive an IUD. Only a veterinarian would apply IUDs in any BLM management action. The IUD is inserted into the uterus using a thin, tubular applicator similar to a shielded culture tube, and would be inserted in a manner similar to that routinely used to obtain uterine cultures in domestic mares. If a mare has a zygote or very small, early phase embryo, it is possible that it will fail to be detected in screening, and may develop further, but without causing the expulsion of the IUD. Wild mares with IUDs would be individually marked and identified, so that they can be monitored occasionally and examined, if necessary, in the future, consistent with other BLM management activities.

Using metallic or glass marbles as IUDs may prevent pregnancy in horses (Nie et al., 2003) but can pose health risks to domestic mares (Turner et al., 2015,; Freeman and Lyle, 2015). Marbles may break into shards (Turner et al., 2015), and uterine irritation that results from marble IUDs may cause chronic, intermittent colic (Freeman and Lyle, 2015). Metallic IUDs may cause severe infection (Klabnik-Bradford et al., 2013).

In domestic ponies, Killian et al. (2008) explored the use of three different IUD configurations, including a silastic polymer O-ring with copper clamps, and the "380 Copper T" and "GyneFix" IUDs designed for women. The longest retention time for the three IUD models was seen in the "T" device, which stayed in the uterus of several mares for 3-5 years. Reported contraception rates for IUD-treated mares were 80%, 29%, 14%, and 0% in years 1-4, respectively. They surmised that pregnancy resulted after IUD fell out of the uterus. Killian et al. (2008) reported high levels of progesterone in non-pregnant, IUD-treated ponies.

Soft or flexible IUDs may cause relatively less discomfort than hard IUDs (Daels and Hughes 1995). Daels and Hughes (1995) tested the use of a flexible O-ring IUD, made of silastic, surgical-grade polymer, measuring 40 mm in diameter; in five of six breeding domestic mares tested, the IUD was reported to have stayed in the mare for at least 10 months. In mares with IUDs, Daels and Hughes (1995) reported some level of uterine irritation but surmised that the level of irritation was not enough to interfere with a return to fertility after IUD removal.

More recently, several types of soft or flexible IUDs have been tested for use in breeding mares. When researchers attempted to replicate the O-ring study (Daels and Hughes 1995) in an USGS / Oklahoma State University (OSU) study with breeding domestic mares, using various configurations of silicone O-ring IUDs, the IUDs fell out at unacceptably high rates over time scales of less than 2 months (Baldrighi et al., 2017; Lyman et al., 2021). Subsequently, the USGS / OSU researchers tested a Y-shaped IUD to determine retention rates and assess effects on uterine health; retention rates were greater than 75% for an 18-month period, and mares returned to good uterine health and reproductive capacity after removal of the IUDs (Holyoak et al., 2021). These Y-shaped silicone IUDs are considered a pesticide device by the EPA, in that they work by physical means (EPA, 2020). The University of Massachusetts has developed a magnetic IUD that has been effective at prolonging estrus and preventing pregnancy in domestic mares (Gradil et al., 2019; Joonè et al., 2021; Gradil et al., 2021; Hoopes et al., 2021). After insertion in the uterus, the three subunits of the device are held together by magnetic forces as a flexible triangle. A metal detector can be used to determine whether the device is still present in the mare. In an early trial, two sizes of those magnetic IUDs fell out of breeding domestic mares at high rates (Holyoak et al., unpublished results), but more recent trials have shown that the magnetic IUD was retained even in the presence of breeding with a fertile stallion (Hoopes et al., 2021). The magnetic IUD was used in two trials where mares were exposed to stallions, and in one where mares were artificially inseminated; in all cases, the IUDs were reported to stay in the mares without any pregnancy (Gradil, 2019; Joonè et al., 2021; Gradil et al., 2021; Hoopes et al., 2021).

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